TRIGEM ANTI-IDIOTYPE MONOLONAL ANTIBODY VACCINE TREATMENT FOR STAGE III MELANOMA: RESULTS OF A MULTICENTER PHASE II TRIAL

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"Running Head":
Anti-idiotype antibody vaccine for stage III melanoma

Purpose: To determine whether treatment with an anti-idiotype monoclonal antibody vaccine (TriGem) that mimics GD2 disialoganglioside is an effective adjuvant treatment for stage III melanoma.

Patients and Methods: Eligible patients had regional lymph node metastases (AJCC stage III melanoma). Following lymphadenectomy, patients were treated with TriGem vaccine weekly for 4 weeks then monthly until disease recurrence.

Results: Sixty-four patients were enrolled and 24 of whom also received high dose interferon Alfa-2b (HDI). At a median follow-up of 2 years, the overall survival (OS) and relapse-free survival (RFS) for all patients were 92% and 72% respectively. OS for patients treated with TriGem alone was 90% and for HDI plus TriGem was 96%. RFS for patients treated with TriGem was 67% and for HDI plus TriGem was 80%. All patients, including those treated with HDI plus TriGem, generated robust anti-GD2 immune responses. Toxicity of the vaccine was primarily limited to local reaction with swelling and pruritis.

Conclusion: Adjuvant therapy with TriGem leads to excellent anti-GD2 immune responses in all patients and appears to produce a clinical benefit in stage III melanoma, particularly for patients treated with HDI plus TriGem. A prospective randomized trial for stage III melanoma patients treated with HDI versus HDI plus TriGem is planned.

INTRODUCTION

Melanoma poses an increasingly important health problem. The incidence of melanoma has increased 10 fold in the past half century with annual rates now approaching 40 cases per 100,000 people in some populations (1). This has resulted in it now being the fourth most common cancer in Australia and New Zealand, and the seventh most common cancer in the United States and Canada. The melanoma annual mortality rates vary from one to three per 100,000 people per year in the northern hemisphere to between five and 10 per 100,000 per year in the southern hemispheric countries such as Australia and New Zealand.

The groups of patients that have conventionally been considered for adjuvant therapy are those at high risk for recurrence with thick primary lesions (>4mm in depth) and/or positive lymph nodes, with a relapse/mortality risk exceeding 50% at 5 years. In an effort to improve the cure rates for locally advanced surgically curable melanoma, several approaches to post surgical adjuvant therapy have been tested over the past three decades. These have included chemotherapy, immune modulation with Bacillus Calmette Guerin (BCG), Corynebacterium parvum, levamisole, and more recently the use of biologic agents such as interferon Alfa-2b and granulocyte macrophage-colony stimulating factor (GM-CSF, 2). The results of randomized clinical trials testing these approaches were uniformly negative until the results of a large cooperative group study testing the use of high dose interferon Alfa-2b (HDI) in patients with high risk stage II and III disease were reported (3). A second trial confirmed a significantly better RFS for patients treated with HDI over a low dose interferon Alfa-2b regimen and an observation arm (4). The most recent phase III trial compared HDI with a ganglioside vaccine (GM2-KLH/QS21) in patients with high-risk resected stage IIb and III melanoma. The results of this trial confirm the benefit of HDI (5).

Gangliosides are sialic acid containing glycosphingolipids that have increased surface membrane expression on malignant melanoma. A major limitation of vaccination with gangliosides has been the requirement to covalently link the ganglioside to keyhole limpet hemocyanin mixed with a potent adjuvant to produce more robust immunoglobulin responses. Another limitation of gangliosides is their expense and difficult purification process. An alternative approach to the natural ganglioside is the generation of anti-idiotype antibodies that mimic the ganglioside. The idiotype network hypothesis of Lindenmann (6) and Jerne (7) offers an elegant approach to transforming epitope structures into idiotypic determinants expressed on the surface of antibodies. According to the network concept, immunization with a given tumor-associated antigen (TAA) will generate production of antibodies against this TAA that are termed Ab1. Ab1 are then used to generate a series of anti-idiotype antibodies against the Ab1, termed Ab2. Some of these Ab2 molecules can effectively mimic the three-dimensional structure of the TAA identified by the Ab1. These particular antibodies called Ab2β, fit into the paratopes of Ab1 and express the internal image of the TAA. The Ab2β can induce specific immune responses similar to those induced by the original TAA and can therefore be used as surrogate TAAs. Immunization with Ab2 can lead to the generation of anti-anti-idiotypic antibodies (Ab3) that recognize the corresponding original TAA identified by the Ab1. Because of this Ab1-like activity, the Ab3 is also called Ab1′ to indicate that it might differ in its other idiotopes from Ab1.

We have been interested in the anti-idiotype approach to a number of antigens (8-17) and have generated an anti-idiotope antibody, designated TriGem, that mimics disialoganglioside GD2 (13-16), which is highly expressed on melanoma and other neuroectodermal tumors, with only minimal expression on normal tissues. In a previous trial, we reported a prolonged overall

median survival (over 16 months) of 47 patients with advanced metastatic melanoma treated with TriGem (16). We demonstrated that hyperimmune sera from 40 of 47 patients generated anti-anti-idiotypic responses that were truly Ab1′ responses based on binding to purified disialoganglioside GD2. We now present the results of a multicenter phase II trial in which patients with stage III melanoma were treated with TriGem following lymph node dissection. The purpose of this phase II trial was to determine RFS, OS and immune responses. In addition, we compared a number of adjuvants including TriGem mixed with QS-21, GM-CSF and TriGem precipitated in aluminum hydroxide (AluGel).

PATIENTS AND METHODS

Patients

All patients had American Joint Committee on Cancer stage III melanoma. Eligible patients must have had a curative resection of the primary lesion and lymph node metastases. Patients who had a positive sentinel lymph node biopsy for melanoma had a complete lymphadenectomy. Patients were enrolled onto the study within 8 months after definitive lymphadenectomy. Patients were allowed onto the study if they received or were on HDI. Patient requirements included a hemoglobin value of 10.5 g/100 ml or greater; renal and hepatic function less than 1.5 times upper limit of normal. All patients had no evidence of distant metastases on bone scan, or computed tomography (CT) of brain, chest, abdomen, and pelvis. Signed informed consent was obtained from all patients.

Treatment Schedule

Initial patient evaluations included a medical history and physical examination; measurement of performance status, complete blood count, renal and hepatic profiles; CT of brain, chest, abdomen, and pelvis, and bone scan. Patients underwent these evaluations at six-month intervals over the next one to five years. All patients were treated subcutaneously weekly for 4 weeks, and then monthly until disease recurrence with TriGem mixed with either 100 μg QS-21, or 100 μg GM-CSF or precipitated in aluminum hydroxide (AluGel).

Statistical Methods

Survival and disease recurrence of all patients were measured from the time of diagnosis. RFS was measured from diagnosis until death or disease recurrence. Survival and RFS were estimated by the Kaplan-Meier method.

Generation of Anti-Idiotype Antibody (TriGem)

The murine monoclonal IgG2a anti-ganglioside GD2 antibody 14.G2a (Ab1) was used to immunize syngeneic BALB/c mice (18). Hybridoma fusion, cloning, and selection of the monoclonal anti-idiotype TriGem (Ab2) as well as production of ascites in bulk quantities in mice were performed as previously described. TriGem (Titan Pharmaceuticals Inc, South San Francisco, CA) was purified form ascites by affinity chromatography on a protein A-CL Sepharose (Pharmacia, Uppsala, Sweden) 4B column followed by diethylaminoethyl ion-exchange chromatography. The purity of the isolated immunoglobulin (> 99%) was determined by sodium dodecyl sulfate polyacrylamide gel electropheresis, high-pressure liquid chromatography, and isoelectric focusing.

Inhibition of Ab2 Binding to Ab1

Preimmune and hyperimmune patients' sera samples were treated with normal mouse immunoglobulin to remove anti-isotypic and anti-allotypic reactivities. Serial dilutions of sera were then tested for inhibition in the Ab1-Ab2 binding assay. All assays were performed in triplicate. For direct binding inhibition between Ab1 and Ab2, purified Ab1 14.G2a was used to coat the plates (500 ng/well), and the binding of radiolabeled TriGem (Ab2) to Ab1 was tested for inhibition in the presence of hyperimmune Ab3 sera from patients. Results from this assay indicated whether the Ab3 in patients' sera shared idiotypes with 14.G2a (Ab1). In addition, this inhibition assay demonstrated whether the Ab3 was a true anti-anti-idiotype.

Immune Flow Cytometric Analysis With Ab1 and Patient's Ab3 Sera

GD2-positive M21/P6 human melanoma cells and GD2-negative LS174T human colon cancer cells (5x 10^5 per tube) were reacted with Ab1 (14.G2a) or patients' Ab3 sera (1:10 dilution) at 4°C for 2 hours. After washing, the cells were incubated with either goat antimouse or goat antihuman IgG/fluorescein isothiocyanate-labeled antibody (Biosource, Camarillo, CA) for 30 minutes at 4°C. The cells were washed with cold phosphate-buffered saline (PBS), fixed in 3% paraformaldehyde, and analyzed by flow cytometry (FACStar; Becton Dickinson, San Jose, CA).

Purification of Anti-Anti-Idiotypic Antibodies (Ab3) From Hyperimmune Patients' Sera

Ab3 was purified from the sera of immunized patients by an immunoadsorbent column consisting of TriGem coupled to Sepharose 4B. Protein bound to this column was eluted with glycine-HCl, pH 2.7, neutralized to pH 7.0 with 3 mol/L Tris and dialyzed against PBS. This material was then passed over a mouse immunoglobulin immunoadsorbent column of Sepharose

4B to remove anti-isotypic and antiallotypic reactivities. Antibody that passed through this column was concentrated and used as purified Ab3.

Determination of Immunoglobulin Isotypes and Subclasses

Microtiter plates were coated with 50 μL of purified GD2 (2 μg/mL) per well and treated with 1 μg of purified Ab3 from each patient. The reaction was developed by enzyme-linked immunoadsorbent assay using anti-isotype-specific reagents. Numbers represent the mean optical density of 450 nm of triplicate wells.

GD2-Binding Inhibition Assay

Purified GD2 antigen (500 ng/well) was coated onto a 96-well plate. After blocking with 1% bovine serum albumin in PBS, different dilutions of purified Ab3 or Ab1 along with radiolabeled 14.G2a antibody (~90,000 cpm) were added to the plates, and the mixtures were incubated for 4 hours at room temperature. After washing, bound radioactivity was measured.

Binding of Ab3 to Purified GD2 and GD3

Anti-GD2 reactivity was determined in the purified Ab3 with GD2 and GD3 that were each adsorbed onto 96-well microtiter plates (250 ng/well). After blocking with 1% bovine serum albumin in PBS, test samples and Ab1 were added to plates at different dilutions and incubated for 4 hours at room temperature with shaking. After washing, the bound antibodies were detected, with alkaline phosphatase-conjugated goat antihuman IgG and goat antimouse IgG serving as second antibodies. The isotype of the antibodies was determined with anti-isotype-

specific reagents (Southern Biotech, Birmingham, AL) such as goat antihuman IgG (γ-chain specific) and goat antihuman IgM (μ-chain specific).

RESULTS

Clinical Results

Sixty-four patients with AJCC stage III melanoma were entered onto this trial. Clinical characteristics of these patients are summarized in Table 1. Twenty-four patients also received HDI in addition to TriGem. All patients entered onto this trial were offered HDI but most of them were referred for TriGem because they refused HDI. The median time for those patients who were treated with HDI from diagnosis to treatment with TriGem was five months. For all other patients, the median time from diagnosis to treatment with TriGem was three months. The OS and RFS for all 64 patients was 92% and 72% respectively at a median follow-up of 24 months (range 2 to 48 months, Figure 1). OS for patients treated with TriGem alone was 90% (4 deaths in 40 patients) and for HDI plus TriGem was 96% (1 death among 24 patients, Figure 2). RFS for patients treated with TriGem alone was 67% and for HDI plus TriGem was 80% (Figure 2). There were 30 patients with N1 disease, 26 with N2 disease and 8 with N3 disease with OS and RFS shown in Figure 3. We also compared OS and RFS in patients treated with TriGem precipitated in AluGel (N=28), TriGem mixed with 100 μg of QS-21 (N=25), and TriGem mixed with 100 μg of GM-CSF (N=16). We found no significant difference in OS and RFS , although there appeared to be a trend of decreased OS and RFS on the QS-21 arm (Figure 4).

Toxicity

Side effects were primarily limited to local swelling at the site of injection; some patients had mild fever and chills. The local reactions were greatest among those patients treated with GM-CSF mixed with vaccine, followed by those treated with vaccine mixed with QS-21, and the least toxic vaccine formulation was with AluGel. There were two grade III toxicities which occurred not during the first injections; two patients, who received the vaccine with GM-CSF, developed generalized hives and were treated with systemic steroids and antihistamines. These two patients withdrew from the study but are included in the data analysis. There were no grade IV toxicities.

Immune Responses

All patients generated active immune responses with high titer anti-GD2 IgG responses and many had measurable CD4 T cell responses. These immune responses persisted throughout the course of therapy. Although there was variation in the quantitative humoral immune response between different patients, the quantity in an individual patient remained constant. Most patients reached a peak anti-GD2 titer after eight vaccinations, and this titer remained constant with monthly booster injections of TriGem. The T cell responses were more variable over time, most likely because of variability of the biologic assay, as it was performed on fresh cells.

It was critical to compare the anti-GD2 immune responses in patients who received HDI with those who did not, because future phase III trials will randomize patients to HDI versus HDI plus TriGem. We found no differences in the anti-GD2 humoral responses in these patients. We studied a representative comparison of six patients who received HDI concurrently with TriGem with six who received TriGem alone. Hyperimmune sera from both groups demonstrated an anti-

anti-idiotype Ab3 response as indicated by the ability of patients' immune sera to inhibit Ab2 binding to Ab1 (Figure 5).

Representative data of inhibition of Ab1 binding to GD2 are shown in Figure 6. Representative data from one non-HDI treated patient showing binding to the GD2-positive human melanoma cell line M21/P6 by immune flow cytometry is shown in Figure 7. Similar results were obtained with patients treated with TriGem plus HDI. Quantitation of purified Ab3 from serum was performed on selected patients, demonstrating a range of 140-470 μg/ml serum, with somewhat higher concentrations in patients treated with HDI . We also studied the isotype subclasses of the anti-GD2 antibodies from patients immunized with TriGem versus TriGem plus HDI and found that IgG was the major immunoglobulin in both groups (data not shown). There were only minor differences in the IgG subclasses; there appeared to be greater IgG1 responses in patients treated with TriGem plus HDI (data not shown). Antibody-dependent cell mediated cytotoxicity was identified in those patients tested, with no differences between those treated with or without HDI (data not shown). Purified Ab3 from sera were also tested against GD2 and GD3 by ELISA and reacted specifically with GD2 with minor reactivity to control GD3 (data not shown). No major differences were found between patients treated with TriGem versus TriGem plus HDI. There appeared to be a modest increase in anti-GD2 antibody activity in the HDI treated patients, which needs to be verified in a larger number of patients. Overall, the six patients in both groups had comparable qualitative and quantitative immune responses.

We also compared immune responses in patients treated with TriGem plus QS-21, TriGem precipitated in AluGel versus TriGem plus GM-CSF. We found no difference in the inhibition of Ab2 binding to Ab1 by patients' immune sera, nor did we find any difference in inhibition of Ab1 binding to disialoganglioside GD2 by patients' immune sera (Figure 8 and 9).

DISCUSSION

Our data suggests that 100% of stage III melanoma patients treated with TriGem generate a robust anti-disialoganglioside GD2 IgG immune response that is not negatively impacted, and may be enhanced by HDI. We also determined that there was no immunologic advantage to any of the three adjuvants tested including QS-21, GM-CSF, and AluGel. RFS and OS for TriGem alone would appear to be comparable to HDI, and the results of RFS and OS of TriGem in combination with HDI suggests an additive or synergistic benefit. However, the delay in treating patients with TriGem who received HDI (median of five months), improved RFS of these patients, because patients who developed recurrent disease within the first few months of treatment with HDI would have been excluded from our study. However, this delay in initiating TriGem would have minimal effect on OS. This additive/synergistic benefit will be the focus of a phase III trial where stage III melanoma patients will be randomized to HDI versus HDI plus TriGem.

The preliminary results of the most recent phase III Intergroup trial (E1694) comparing HDI with the GMK ganglioside vaccine (GM2-KLH/QS-21) in patients with high risk resected stage IIb and III melanoma have been published (5). The trial was closed in May, 2000 due to a significant benefit of HDI over GMK both in terms of RFS (RFS HR 1.47, p1 = 0.0015, p2 = 0.003) and OS (OS HR 1.92, p1 = 0.009, p2 = 0.018). There was no evidence that GMK adversely affected RFS or OS based on prior intergroup control arms. The two-year analysis from this trial (personal communication, Dr. John Kirkwood) of 440 patients treated with HDI demonstrated OS and RFS of 78% and 68% respectively. In our study, the OS and RFS of 24

patients treated with HDI plus TriGem was 96% and 80% respectively at 2 years. While our numbers are small, these data suggest a benefit of combining HDI plus TriGem.

A variety of agents have been studied in patients with high risk melanoma. GM-CSF activates macrophages, stimulates monocytes in vitro to become cytotoxic to melanoma cells (19,20), increases the cytotoxicity of monocytes in vivo (21, 22) and mediates proliferation, migration and maturation of dendritic cells (23-25). In addition, GM-CSF also stimulates production of angiostatin by macrophages (26). Forty-eight patients with resected stage III and IV melanoma were treated with GM-CSF administered subcutaneously in 28 day cycles (14 days of GM-CSF followed by 14 days off therapy.) Cycles continued for one year or until disease recurrence. The median survival of resected stage IV patients was 37.5 months versus 12.2 months ($p > 0.001$) in matched controls (27). For the 14 patients with high-risk stage III disease, the overall median survival was 35.8 months compared to 15.2 months for matched controls ($P>0.04$). These results are intriguing, however they require confirmation in prospective randomized phase III trials.

A variety of other vaccines are being studied in phase III trials. Autologous tumor cells modified with dinitrophenyl plus BCG have demonstrated promising results (28-30). CancerVax is a viable antigen enriched melanoma cell vaccine developed from three melanoma cell lines. Phase II trials of CancerVax in resected stage IV and stage III melanoma patients have suggested improved survival that correlated with immune responses and randomized phase III studies are ongoing (31-34). Allogeneic melanoma lysates have been used as active immunotherapy and are derived from whole melanoma cell lines. Melacine is a lyophilized preparation from two melanoma cell lines (35-37). The most recent phase III trial from The Southwest Oncology Group for patients with resected node negative melanomas suggested some benefit in certain patient subsets (personal communication). Polyvalent shed antigen vaccine is prepared from

material shed by viable tumor cells into culture medium from four melanoma cell lines. Non-randomized trials of this vaccine in patients with surgically resected AJCC stage II and stage III melanoma suggested both humoral and cellular responses in 50% of patients and improved survival that correlated with these immune responses (38-40). A variety of other vaccines including autologous melanoma cells engineered to secrete human GM-CSF, a variety of peptide vaccines as well as dendritic cell vaccines are being studied (41-52).

Gangliosides are neuraminic acid containing glycosphingolipids that are anchored into the lipid bilayer of the plasma membrane. The carbohydrate portion of gangliosides is present on the extracellular border of the plasma membrane. They are overexpressed in tissues of neuroectodermal origin and particularly in tumors such as melanomas, neuroblastomas and sarcomas. One-hundred and twenty patients with AJCC stage III melanoma were randomized to receive either GM2/BCG vaccine or BCG alone (53). All patients were pretreated with low dose cyclophosphamide. There was significantly increased relapse-free survival and overall survival for those patients who generated an antibody titer against GM2 of greater, or equal to 40 compared with antibody negative patients. To improve the humoral response, GM2 was conjugated to keyhole limpet hemocyanine and administered with the QS-21 adjuvant. However, the results of the phase III trial comparing this vaccine to HDI demonstrated the superiority of HDI (5). We have chosen an alternative approach to the natural ganglioside using an anti-idiotype antibody that conformationally mimics the disialoganglioside GD2. One anti-idiotype antibody, BEC-2, which putatively mimics disialoganglioside GD3 is currently being investigated in clinical trials. However, anti-GD3 responses have not commonly been reported in patients immunized with BEC-2 (54,55). The major differences between TriGem and GM2-KLH/QS-21 and BEC-2 are the immune responses. TriGem generates robust immune responses

in nearly 100% of patients and the immune responses are predominantly IgG, including all subclasses that bind to purified GD2 (titers over 1:1000) and GD2 positive cells by immune flow cytometry, and mediate ADCC. BEC-2, as mentioned above, which is an anti-idiotype that mimics the disialoganglioside GD3, generates specific anti-GD3 immune responses in only a minority of patients while GM2-KLH/QS21, which is a purified ganglioside vaccine, generates specific immune responses in about 80% of patients.

In conclusion, our data suggest a clinical benefit of TriGem in stage III melanoma, particularly for patients treated with HDI plus TriGem. A prospective randomized trial for stage III melanoma patients treated with HDI versus HDI plus TriGem is planned.

Acknowledgements.

This work was supported in part by the following grants from the National Institutes of Health (NIH): R01 CA-72018; R01 CA-72773 and R01 CA-86025. We would like to thank Karen Ashbrock for typing the manuscript.

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Table 1. Patient Characteristics

	No. of Patients	% of Total
Age, years		
Median 52		
Range 17-86		
Sex		
Male	44	69
Female	20	31
N Stage		
N1 (1 node)	30	46
N2 (2-3 nodes)	26	41
N3 (4 or more)	8	13
High-dose Interferon		
No	40	62
Yes	24	38
TriGem mixed with:		
AluGel	28	43
GM-CSF	15	24
QS-21	21	33

Legends for Figures:

Figure 1. Kaplan-Meier overall survival (A) and relapse-free survival (B) of all 64 patients.

Figure 2. Kaplan-Meier overall survival (A) and relapse-free survival (B) of patients treated with TriGem plus HDI (●-●; 24 patients) versus TriGem alone (■-■; 40 patients).

Figure 3. Kaplan-Meier overall survival (A) and relapse-free survival (B) of all patients according to N stage; (N1 ●-●), (N2 ■-■), and (N3 ▲-▲).

Figure 4. Kaplan-Meier overall survival (A) and relapse-free survival (B) of all patients according to adjuvant type; (Alugel ●-●), (GM-CSF ■-■), and (QS-21 ▲-▲).

Figure 5. Inhibition of Ab2 (TriGem) binding to Ab1 (14.G2a) by patients' immune sera. Purified Ab1 was used to coat the plate, and binding of radiolabeled Ab2 to Ab1 was tested for inhibition in the presence of patients' immune sera. A: patients treated with TriGem plus HDI. B: patients treated with TriGem only.

Figure 6. Inhibition of Ab1 (14.G2a) binding to ganglioside GD2 by patients' immune sera. GD2 was used to coat the plate and binding of radiolabeled Ab1 to GD2 was tested for inhibition in the presence of patients' hyperimmune sera (between 14x-18x) at a 1:10 dilution. A: patients treated with TriGem plus HDI. B: patients treated with TriGem only.

Figure 7. Immune flow cytometry of GD2 positive and negative cells with purified Ab3. A: GD2 positive cells were reacted with the preimmune IgG and immune Ab3 (1μg). B: GD2 negative cells were reacted with same antibodies. C: GD2 positive cells were reacted with PBS control and Ab1, 14.G2a. Representative data from one patient is presented.

Figure 8. Inhibition of Ab2 (TriGem) binding to Ab1 (14.G2a) by patients' immune sera. Ab1 was used to coat plate, and binding of radiolabeled Ab2 to Ab1 was tested for inhibition in the presence of different dilutions of immune sera (between 14x-17x). Patients treated with A: TriGem+QS-21; B: TriGem+AluGel; C:TriGem+GM-CSF.

Figure 9. Inhibition of Ab1 (14.G2a) binding to ganglioside GD2 by patients' immune sera. GD2 was used to coat the plate and binding of radiolabeled Ab1 to GD2 was tested for inhibition in the presence of a 1:10 dilution of patients' immune sera (between 14x-17x). Patients treated with A: TriGem+QS-21; B: TriGem+AluGel; C: TriGem+GM-CSF.